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EFFECT OF ACUTE ETHANOL ADMINISTRATION ON THE SUBCELLULAR DISTRIBUTION OF IRON IN RAT LIVER AND CEREBELLUM

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Abstract—An acute ethanol load (50 mmol/kg, i.p.) produced altogether a decrease in the non-heme iron content of the serum and an increase in the iron content in liver and cerebellum. Subcellular fractionation studies indicated that the non-heme iron accumulated by the liver, 4 hr after the ethanol load, was recovered in light mitochondria, microsomes and cytosol, and that iron accumulated by the cerebellum was localized in heavy mitochondria, light mitochondria, microsomes and cytosol. The low molecular weight chelatable (LMWC) iron content as well as the percentage of total non-heme iron represented by LMWC-iron were increased in the cytosol of liver and cerebellum after the ethanol load. These results suggest that an acute ethanol load induces (i) a shift in the distribution between circulating and tissular non-heme iron; (ii) an increase in the cytosolic LMWC-iron which, by favouring the biosynthesis of reactive free radicals, may contribute to lipid peroxidation in liver and cerebellum.

Several reports have been concerned with the effects of an acute ethanol intoxication on hepatic lipid peroxidation. Following a single dose of ethanol, evidence has been found for increased levels of malondialdehyde [1] and of conjugated dienes [2] in the liver as well as for increased alkane exhalation [3]. Although lipid peroxidation is not likely to be the sole cause of ethanol hepatotoxicity, most results suggest that the process can undoubtedly contribute to this toxicity. Moreover our previous data have shown that the enhancement in lipid peroxidation induced by an acute ethanol load is not restricted to the liver [4], but affects also the cerebellum [5]. These changes in lipid peroxidation reflect an oxidative stress, i.e. a disturbance in the pro-oxidant/antioxidant systems in favour of the former [6].

Oxygen-derived free radicals, such as the hydroxyl radical ($^{\cdot}OH$), are to be considered as important cellular pro-oxidants. Following ethanol administration $^{\cdot}OH$ may be issued from $O_2^{\cdot-}$ and H_2O_2 generated during the metabolism of ethanol and acetaldehyde by secondary pathways [7] or through the mitochondrial respiratory chain [8]. The biosynthesis of $^{\cdot}OH$ is catalysed by transition metals, especially iron. A fraction of the cellular non-heme iron consisting in low molecular weight chelatable iron derivatives (LMWC-iron) appears to represent the iron species catalytically active in initiating free radical reactions and lipid peroxidation [9–11]. This fraction can be considered as decompartmentalized iron, contrarily to iron bound to proteins such as ferritin or hemosiderin.

The aims of the present study were to determine whether an acute ethanol load affects: (i) the distribution between circulating and tissular total non-heme iron; (ii) the subcellular distribution of non-heme iron in the liver and cerebellum and (iii) the LMWC-iron content of tissular cytosolic fractions.

MATERIALS AND METHODS

Chemicals. The iron standard, 1000 ppm in nitric acid was from Merck (Darmstadt, F.R.G.). Desferrioxamine methane sulfonate (desferal^{R}) was a gift from Ciba-Geigy (France). Chelex-100 was from Bio-Rad Laboratories (Richmond, CA). All other chemicals were of analytical purity.

Animals and treatments. Male Sprague–Dawley rats weighing about 180 g were fed a standard pellet diet (Iffa Credo, UAR) containing 100 ppm iron. Except when otherwise stated, 50 mmoles of ethanol per kg body wt, were administered i.p. as a 20% (v/v) aqueous solution (1.45 mL/100 g body wt) to overnight fasted animals. The same volume of saline was injected i.p. to the control animals.

After the rats were killed by decapitation, the liver and cerebellum were rapidly removed. A sample of the liver and the cerebellum was washed, mopped up, frozen and kept in liquid nitrogen until used for the analysis of the non-heme iron content.

Subcellular organelle fractionation. For the preparation of the liver subcellular fractions, the liver was finely minced and homogenized in 5 vol. ice-cold 0.33 M sucrose, pH 7.4, by 20 strokes in a Potter homogenizer with a motorized rotating Teflon pestle. The subcellular fractions of rat liver were prepared essentially as described in Ref. 12. The protein content of nuclei, heavy mitochondria, light mitochondria, microsomes and cytosol obtained by this

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procedure was 56.2 ± 3.6; 36.2 ± 2.5; 18.2 ± 1.0; 27.4 ± 2.8; 72.2 ± 3.8 mg/g wet wt tissue (N = 5), respectively, the average recovery of total liver protein being 100.1%. The cerebellum was washed and homogenized in 10 vol. ice-cold 0.33 M sucrose, pH 7.4. The total cerebellar homogenate was submitted to differential centrifugation by a five-fraction scheme according to Ref. 13. The protein content of nuclei, heavy mitochondria, light mitochondria, microsomes and cytosol prepared by this procedure was 26.6 ± 1.8; 28.7 ± 1.5; 15.5 ± 3.1; 21.0 ± 2.0 and 42.6 ± 3.3 mg per g wet wt tissue (N = 5), respectively, the average total recovery of cerebellar proteins being 99.7%.

Preparation of low molecular weight filtrates. Preparation of low molecular weight filtrates was achieved by filtration of samples of the liver and cerebellar cytosolic fractions through a YMT ultrafiltration membrane in an Amicon-MPS 1 device (cut off 30,000 daltons) [14]. No retention of iron by the ultrafiltration membrane was observed when submitting desferal–iron samples to the ultrafiltration device. Furthermore the recovery of desferal–iron added to cytosolic samples through the membrane was greater than 95%.

Chemical analyses. For the determination of non-heme iron in tissues and subcellular fractions following trichloracetic and hydrochloric acid extraction two methods were used: (i) colorimetric determination [15]; (ii) analysis by inductively coupled plasma atomic emission spectrometry (I.C.P.A.E.S.) according to Ref. 16. Both methods gave comparable results for tissular non-heme iron. Since the LMWC-iron level present in tissular extracts is very low, the results reported in this communication were obtained with the I.C.P.A.E.S. method which is characterized by its high sensitivity (2 ng/mL). Protein was assayed following Lowry *et al.* [17]. All solutions were prepared with Chelex 100-treated water. Blood ethanol concentrations were determined by gas chromatography [18].

Statistical interpretation. Reported values are means ± SE, statistical analysis being performed by using Student's *t*-test.

RESULTS

Following the acute ethanol administration (50 mmol/kg body wt, i.p.) to fasted rats, the mean blood ethanol concentration for nine animals was 50 ± 2, 40 ± 3, 27 ± 2 mM after 2, 4 and 6 hr of treatment, respectively. No ethanol could be detected in the blood 24 hr after the ethanol load.

This ethanol treatment resulted in a significant decrease in the circulating non-heme iron level decrease which was apparent from 2 hr and remained constant 4, 6 and 24 hr after the ethanol load (Table 1). In contrast to the decrease observed in the serum, the non-heme iron content was significantly increased in the liver and cerebellum. This increase was apparent 2 hr after ethanol and reached a plateau level later on. However the ethanol load did not modify the non-heme iron level in the brain cortex during the period studied (results not shown).

Using fed rats instead of fasted ones, similar results were obtained 4 hr after ethanol (results not shown).

In order to evaluate the threshold ethanol dose which produces changes in iron distribution, fasted rats received an i.p. ethanol load at a dose of either 25 or 37.5 mmol/kg body wt. No changes were observed in the non-heme iron distribution in the tissues studied 4 hr after the lowest ethanol dose, the blood alcohol concentration at that time being 2.8 ± 0.3 mM. However 4 hr after the i.p. administration of 37.5 mmol/kg body wt the serum non-heme iron was significantly ($P < 0.01$) decreased (−22%) and the hepatic and cerebellar non-heme iron levels significantly ($P < 0.05$) increased (+15% and +12%, respectively). The blood ethanol concentration in these animals was 18.5 ± 4 mM (N = 4).

To test whether the observed changes were dependent on the administration route, oral administration of ethanol (108 mmol/kg body wt) was performed to fasted rats. Using a 25.3% (v/v) ethanol solution (2.5 mL/100 g body wt) both a significant decrease in serum non-heme iron and a significant increase in liver non-heme iron were observed 90 min after the ethanol load (17.3 ± 1.8 vs 11.1 ± 2.2 μmol/L

Table 1. Non-heme iron levels in blood serum, liver and cerebellum after an acute ethanol load

Time (hr)	2	4	6	24
Serum				
Control	14.5 ± 3.8 (9)	14.1 ± 1.6 (15)	14.5 ± 1.1 (9)	14.8 ± 0.5 (6)
Ethanol	9.2 ± 0.8 (7)*	8.7 ± 2.4 (16)*	8.2 ± 1.3 (7)*	9.8 ± 0.3 (6)*
Liver				
Control	1.76 ± 0.07 (9)	1.96 ± 0.09 (10)	1.78 ± 0.07 (9)	1.78 ± 0.03 (6)
Ethanol	2.18 ± 0.16 (7)*	2.50 ± 0.03 (11)*	2.28 ± 0.25 (9)*	2.18 ± 0.03 (6)*
Cerebellum				
Control	0.151 ± 0.003 (9)	0.139 ± 0.003 (19)	0.151 ± 0.003 (19)	0.153 ± 0.003 (9)
Ethanol	0.214 ± 0.009 (7)*	0.187 ± 0.005 (20)*	0.178 ± 0.003 (19)*	0.191 ± 0.002 (6)*

Ethanol (50 mmol/kg) was injected i.p. into overnight fasted rats. Values are expressed as μmol/L serum or μmol/g liver or cerebellum (wet wt). Values are means ± SE. Number of animals in parentheses.

Statistical significance: *$P < 0.01$ versus controls.

Table 2. Effect of an acute ethanol load on the hepatic subcellular distribution of non-heme iron

Fraction	Non-heme iron (nmol/mg protein)		Difference (%)
	Controls	Ethanol-treated rats	
H	7.9 ± 0.2	9.7 ± 0.3	+23*
N	5.1 ± 0.2	5.4 ± 0.2	+5
M	6.0 ± 0.5	5.5 ± 0.5	−8
L	7.2 ± 0.2	9.0 ± 0.4	+25*
P	10.3 ± 0.4	17.7 ± 0.7	+72*
S	4.9 ± 0.3	6.9 ± 0.2	+39*

Overnight fasted rats received ethanol (50 mmol/kg, i.p.) 4 hr before being killed. H, homogenate; N, crude nuclear fraction; M, heavy mitochondrial fraction; L, light mitochondrial fraction; P, microsomal fraction; S, cytosolic fraction. The values given are means ± SE of 11 controls and 11 ethanol-treated rats.

* Indicates significantly different from the controls as determined by the Student's *t*-test ($P < 0.001$).

Table 3. Effect of an acute ethanol load on the subcellular distribution of non-heme iron in the cerebellum

Fraction	Non-heme iron (nmol/mg protein)		Difference (%)
	Controls	Ethanol-treated rats	
H	0.99 ± 0.03	1.35 ± 0.03	+36*
N	1.04 ± 0.04	1.04 ± 0.04	0
M	1.13 ± 0.04	1.41 ± 0.05	+24*
L	1.27 ± 0.04	1.63 ± 0.04	+28*
P	1.45 ± 0.08	2.42 ± 0.11	+66*
S	0.37 ± 0.04	0.80 ± 0.05	+116*

Overnight fasted rats received ethanol (50 mmol/kg, i.p.) 4 hr before being killed. Fractions abbreviated as in Table 2. The values given are means ± SE of 11 controls and 11 ethanol-treated rats.

* Indicates significantly different from the control as determined by the Student's *t*-test ($P < 0.001$).

serum, $P < 0.01$ and 1.69 ± 0.07 vs 2.07 ± 0.14 μmol/g liver, $P < 0.01$ in five controls and five ethanol-treated animals, respectively). Similar results were obtained when administering the same ethanol dose as a 50.6% (v/v) ethanol solution (1.25 mL/100 g body wt) (results not shown).

The hepatic subcellular distribution of non-heme iron 4 hr after the ethanol administration (50 mmol/kg body wt, i.p.) to fasted rats is shown in Table 2. An increased non-heme iron content is apparent in the light mitochondrial (+25%), microsomal (+72%) and cytosolic (+39%) preparations. In contrast the non-heme iron level of the crude nuclear and heavy mitochondrial fractions did not show any significant change.

In the cerebellum, the concentration of non-heme iron was increased in all subcellular fractions studied except in the crude nuclear one following the acute ethanol load (Table 3). The magnitude of this increase in heavy mitochondrial, light mitochondrial, microsomal and cytosolic preparations being 24, 28, 66 and 116%, respectively, it appears that the greatest increase in the cerebellar non-heme iron content occurred in the cytosolic fraction.

Analysis of the cytosolic fraction showed a 100% increase in the LMWC-iron content (expressed as nmol/mg protein) in the liver following the ethanol treatment (Table 4). Also of interest was the finding that the percentage of cytosolic non-heme iron represented by LMWC-iron in the liver supernatants appeared to be higher in ethanol-treated rats.

An increase in the cerebellar cytosolic LMWC-iron (+200%) as well as a higher percentage of cytosolic non-heme iron represented by LMWC-iron were also found in the cerebellum of ethanol-treated rats (Table 4).

DISCUSSION

The present results show that an acute ethanol load elicits at the same time a decrease in the non-heme iron content in the serum and an increase in this content in the liver and the cerebellum. This last increase appears within 2 hr and is still apparent 24 hr after the ethanol load, i.e. at a time at which no more alcohol is present in the blood. These results suggest that an acute ethanol load induces a shift in the distribution between circulating and tissular non-heme iron and that the iron taken up by the liver and the cerebellum remains settled in these tissues during 24 hr. It has been previously shown [19] that

Table 4. Effect of an acute ethanol load on the total non-heme iron and the low molecular weight chelatable iron contents of the cytosolic fraction from liver and cerebellum

	Liver		Cerebellum	
	Control	Ethanol	Control	Ethanol
Total non-heme iron (nmol/mg protein)	4.9 ± 0.3	6.9 ± 0.2*	0.37 ± 0.04	0.80 ± 0.05*
Low molecular weight chelatable iron				
(nmol/mg protein)	0.16 ± 0.01	0.32 ± 0.015*	0.020 ± 0.002	0.054 ± 0.002*
(% of cytosolic iron)	3.3 ± 0.2	4.7 ± 0.2*	5.0 ± 0.4	6.6 ± 0.4*

Overnight fasted rats received ethanol (50 mmol/kg, i.p.) 4 hr before being killed. Values are means ± SE of 11 controls and 11 treated rats.

* Indicates significantly different from the control ($P < 0.001$).

iron taken up by the liver from labelled transferrin remains in the tissue for at least 14 days. Few reports [20, 21] have been concerned with the effects of acute ethanol on the non-heme iron content in tissues and conflicting results have been reported. Our data show that a single ethanol administration to fasted or to fed rats, either orally or intraperitoneally, results in a decrease in the circulating non-heme iron and an increase in the cerebellar and hepatic non-heme iron content.

From studies dealing with the mechanisms involved in the iron uptake from transferrin by hepatocytes, it appears that at least two models could account for this process. Firstly the uptake of iron can occur by receptor-mediated endocytosis of transferrin, followed by the release of iron within acidic vesicles and extrusion of the iron-depleted transferrin [22]. Secondly the uptake of iron can also occur at the plasma membrane level without internalization, this process involving labilization and reduction of transferrin-bound iron by co-operative proton and electron fluxes [23, 24]. Since the metabolism of ethanol in the liver results in the production of excess reducing equivalents, NADH may represent the reductant [25] involved in the labilization and reduction of transferrin–iron following ethanol administration.

Ethanol administration increases the cerebellar, but not the cortex, iron content. This finding could be a rationale to understand why the cerebellum is especially sensitive to ethanol compared to several other brain regions. Since transient ischemia may increase both iron and lipid peroxidation [26, 27], it can be suggested that the reduction of cerebellar blood flow linked to acute ethanol administration [28] may contribute to the increase in the cerebellar iron content.

Several reports have been concerned with the subcellular distribution of recently absorbed labelled transferrin–iron. It is apparent from differential and isopycnic centrifugation that iron is rapidly dissociated from transferrin and that a major part of this iron is rapidly incorporated into cytosolic ferritin [19, 22, 29–31]. The distribution of iron into the subcellular organelles is controversial, since it has been reported that iron may be associated with mitochondria [22, 29, 30], microsomes [19] or mitochondria, lysosomes and microsomes [32]. Our results show that the iron apparently taken up by the liver after an acute ethanol load was recovered in light mitochondria, microsomes and cytosol. The major part of this iron being associated with microsomes, it could contribute to the ethanol-induced peroxidation of microsomal lipids [33].

There was no previous report, to our knowledge, concerning the subcellular distribution of iron in the cerebellum. Our results show that the greatest recovery of iron taken up by this tissue after an acute ethanol load was in the cytosol.

Interestingly, the increase in non-heme iron observed in the hepatic and cerebellar cytosolic fractions of ethanol-treated rats was accompanied by an enhancement in the content of LMWC-iron. Numerous studies with ^{59}Fe have reported the presence of a LMWC-iron pool in various cells. Attempts to quantitate this iron pool indicated that it may represent 20% [19], 8% [14] or 6.8% [24] of the total non-heme iron in the cytosol of hepatocytes and 32% [14] in the cytosol of brain tissue. Our results show that LMWC-iron represents only 3.3% and 5% in the cytosol of liver and cerebellum, respectively.

Several *in vitro* studies have reported that reducing agents such as superoxide [34, 35] enhance the release of iron from ferritin. The increase in the percentage of cytosolic LMWC-iron induced by ethanol could therefore be mediated, at least partly, by the resulting cellular reducing environment [36] and/or by enhanced O_2^- production [37] which would favour such a release. Whether the increase in LMWC-iron presently reported in liver and cerebellum after an acute ethanol administration participates to the enhancement in lipid peroxidation that we observed in these tissues following such a load [4, 5] remains to be ascertained. However it is known that many low molecular weight iron complexes are effective initiators of lipid peroxidation *in vitro* [38]. Moreover Bacon *et al.* [39] have reported that hepatic ultrafiltrates prepared from control rats have a pro-oxidant action. Thus the LMWC-iron content of such ultrafiltrates of control rat liver appears sufficient to catalyse free radical reactions. An increase in this amount could favour lipid peroxidation as suggested by Ferrali *et al.* [40] who reported that acrolein (a metabolite of allyl alcohol) altogether releases iron from iron stores and promotes lipid peroxidation in erythrocytes. It can there-

fore be suggested that the increased LMWC-iron content presently reported could contribute to the ethanol-induced oxidative stress in liver and cerebellum by favouring free radical-mediated damaging reactions.

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